泰生科技香港有限公司
N-Cell Technology Hong Kong Limited

PlantNitro® User Manual

Catalog No.: PME-001

N-CELL Technology Hong Kong Limited

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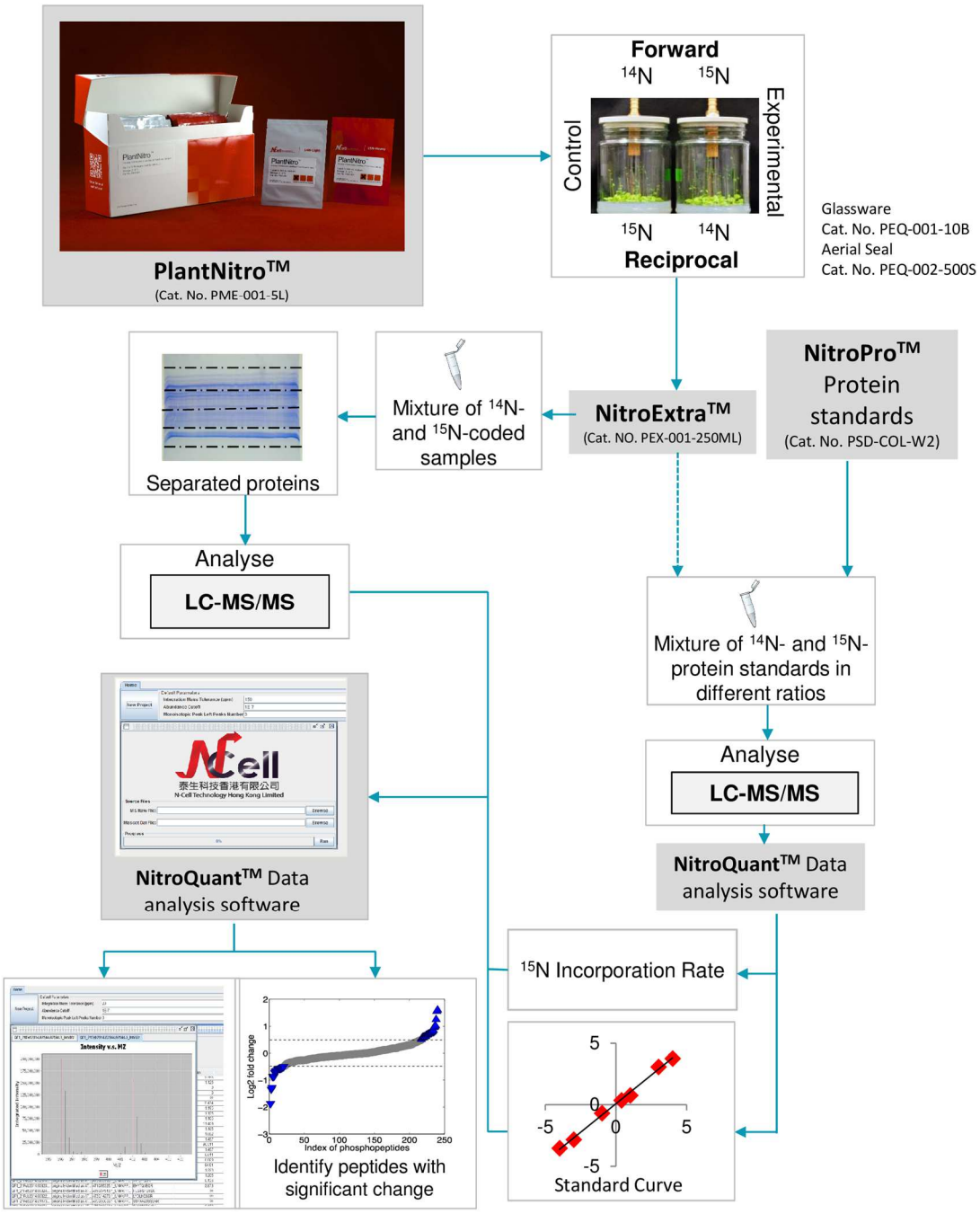
1. Overall Workflow

A brief procedure to perform Nitrogen-15 (15N) stable isotope labeling of Arabidopsis or Brassica is described below. For details on media preparation, planting, protein extraction, protein analysis and mass spectrometry (MS) analysis, refer to the detailed procedures in this manual.



<table>
<tr><th>Step</th><th>Procedure</th></tr>
<tr><td>Seed preparation</td><td>1. Put about 0.2 ml fresh and dry seeds in 1.5 ml micro centrifuge tube.
2. Add 1 ml 70% Ethanol, vortex 1 minute. Spin down the seeds using micro centrifuge.
3. Discard the supernatant, add 1 ml (30% Bleach, 0.1% TritonX-100), and incubate for 5-10 minutes. Spin down the seeds using micro centrifuge.
4. Discard the supernatant, wash with sterile water at least 5 times.
5. After washing, let the seeds dip in sterile water at 4^{O}C in darkness for at least 3 days.</td></tr>
<tr><td>14N-Light and 15N-Heavy medium preparation</td><td>Prepare 14N-Light and 15N-Heavy PlantNitro® medium as following:
<table><tr><th>14N-Light medium</th><th>15N-Heavy medium</th></tr><tr><td>1. Add 500 mL pure (Milli-Q or double distilled) water to 1 package of PlantNitro 14N-Light medium powder, with mixing.
2. Add 10 g sucrose (optional).
3. Adjust the pH to 5.7 (recommended).
4. Add 4 g agar (omit this step if liquid medium is preferred).</td><td>1. Add 500 mL pure (Milli-Q or double distilled) water to 1 package of PlantNitro 15N-Heavy medium powder, with mixing.
2. Add 10 g sucrose (optional).
3. Adjust the pH to 5.7 (recommended).
4. Add 4 g agar (omit this step if liquid medium is preferred).</td></tr></table></td></tr>
</table>

Step	Procedure
Stable isotope labeling	1. Plant appropriate amount of sterilized seeds on the agar surface of the light or/and heavy medium prepared in the pervious step. 2. Incubate in appropriate temperature and lighting depending on the species of the plant for 20-30 days. 3. Perform the appropriate tissue treatments and collect samples. 4. Analyze tryptic peptides using MALDI-TOF MS or LC-MS. Perform protein identification using MS instrument software or Mascot software suite.

2. Contents

The table below lists additional products and service available separately. For more information, visit ncelltech.com or call Technical Support at (+852) 3460 3118 or email to support@ncelltech.com.

Product	Quantity	Catalog no.
NitroExtra™ Protein extraction kit	250 ml	PEX-001-250ML
NitroQuant™ Online Data analysis	Company Webpage	APP-001
NitroPub service	Contact Us	

3. Technical Introduction

The stable isotope labeling of plant is a powerful tool in Systems Biology for quantitative analysis including post-translational modifications, low abundance proteins, phosphoproteins and membrane proteins using heavy nitrogen labeled plant tissues. The PlantNitro® one-step 15N uniform metabolic labeling kit is based on the well established and nutrient rich Murashige & Skoog medium with improved nutritional balance and enhanced high labeling efficiency. Using PlantNitro® medium, the stable isotope nitrogen (15N) is incorporated into proteins in a uniformed manner and enable the confirmation of the identity of proteins with high confidence by comparing the sequence dependent mass shifts of an entire protein digest to the peptide mass fingerprint of the unlabeled protein and residue specific mass alterations. This procedure efficiently detects protein modifications such as phosphorylation.

PlantNitro® as the only broad spectrum labeling method available represents the most powerful tool currently available in the study of quantitative phosphoproteomics.

In uniform nitrogen 15 stable isotopic labeling experiments, two plant populations are grown in identical (except the nitrogen source) plant growing media. One plant population is grown in medium with heavy (isotopic) nitrogen source while another is grown in medium with a light (normal) nitrogen source. The natural metabolic machinery of the cells is utilized to label all cellular proteins with the heavy nitrogen atoms. After trypsin digestion, the peptides containing light or heavy nitrogen are chemically identical and can be processed together using any protein separation method to eliminate quantitation errors due to unequal sampling. Because the peptides are isotopically distinct, they can be easily distinguished by mass using MS analysis. Based on relative peak intensity of the isotopic peptide pairs, one can quantify differential protein expression and identify differential post-translational modifications between samples or in response to a stimulus.

4. Experiment Outline

The experiment outline and general experiment timelines for using the PlantNitro® are shown below.

Step	Action	Page no.	Approx. time taken
A	Sterilize seeds of the model plant	8 – 9	3 days
B	Prepare the 14N-Light and 15N-Heavy PlantNitro® media	8 – 9	2 hours
C	Grow the sterilized seeds as two different populations; grow one plant population in 14N-Light PlantNitro® medium and the other in 15N-Heavy PlantNitro® medium	10	1day
D	Wait for the harvesting of plant tissue	10	Varies
E	Perform cell treatment, if needed	N/A	1-7days
F	Harvest the plant tissue using liquid nitrogen from each population, grind the tissue into fine powder	10	1 hour
G	Mix the tissue powder using equal protein amounts. **OR** Mix the tissue powder using equal tissue amounts.	10	30 minutes

Step	Action	Page no.	Approx. time taken
H	Extract the protein from the tissue using NitroExtra™ protein extraction buffer or other extraction buffers that can extract the protein and maintain the protein in a fully denatured state	10	30 minutes
I	Process the samples using standard protein or peptide separation methods	11 – 12	2-4 days
J	Analyze tryptic peptides using MALDI-TOF MS, LC-MS or Orbit trap	12	2-6 days
K	Preform protein identification using Mascot software suite	13 – 15	3-7 days
L	Preform protein quantitation using NitroQuant™ software	15 – 22	

5. Methods

- The PlantNitro® labeling does not affect the growth of the plants. The plant species choice must be able to grow in Murashige & Skoog medium.
- If you have access to MS core facilities in your research institute, follow the recommended protocols described by them.
- For more technical resources on plant labeling and data analysis, email to support@ncelltech.com .
- If you have an optimized plant culture protocol for your model plant, use the optimized protocol.
- All solutions and equipment that come in contact with the cells must be sterile or autoclaved. Always use proper sterile techniques and work in a laminar flow hood.
- Always use seeds with an ensured germination rate. Testing of the germination rate of your seed stock is recommended.
- Culture plant in the autoclavable glass jar and seal with aerial seal film of appropriate size. These are available from N-Cell (Cat. No. PEQ-001).

Caution:

The PlantNitro® powder and medium is irritating to eyes, skin and respiratory system. In case where contact is made with the eyes rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing.

5.1. Required Materials

- ✓ Fresh seeds of model plant
- ✓ Sterilized water
- ✓ 75% ethanol
- ✓ 30% bleach solution
- ✓ Sterile centrifuge tubes
- ✓ Reagent to determine protein concentration (such as DC-assay)
- ✓ Appropriate reagents for plant treatment if applicable
- ✓ 500 ml autoclavable reagent bottles
- ✓ Autoclavable glass jars for plant culture (Cat. No. PEQ-001)
- ✓ Autoclavable aerial seal film and rubber band (Cat No. PEQ-002)

5.2. Step A: Sterilize seeds of the model plant

Note: If you have an optimized protocol for your model plant, please use your own optimized protocol.

1. Put about 0.2 ml fresh and dry seeds in a 1.5 ml micro centrifuge tube.
2. Add 1 ml 70% ethanol and vortex for 1 minute.
3. Spin down the seeds by brief centrifugation.
4. Discard the supernatant, add 1 ml mixture of 30% Bleach and 0.1% TritonX-100, and incubate for 5-10 minutes.
5. Spin down the seeds by brief centrifugation.
6. Discard the supernatant, wash with sterile water at least 5 times.
7. After washing, let the seeds dip in sterile water at 4 ^{O}C in darkness for at least 3 days.

5.3. Step B: Prepare the 14N-Light and 15N-Heavy PlantNitro® medium

The 14N-Light and 15N-Heavy medium can be prepared according to the table below:

14N-Light medium	15N-Heavy medium
1. Add 500 mL pure (Milli-Q or double distilled) water to 1 package of PlantNitro 14N-Light medium powder, with mixing. 2. Add 10 g sucrose (optional). 3. Adjust the pH to 5.7 (recommended). 4. Add 4 g agar (omit this step if liquid medium is preferred).	1. Add 500 mL pure (Milli-Q or double distilled) water to 1 package of PlantNitro 15N-Heavy medium powder, with mixing. 2. Add 10 g sucrose (optional). 3. Adjust the pH to 5.7 (recommended). 4. Add 4 g agar (omit this step if liquid medium is preferred).

1. Name the reagent bottles as 14N-Light and 15N-Heavy accordingly. Each package of 14N-Light and 15N-Heavy will make up 1 bottle of 500 ml medium. Each bottle will make up 10 plant-culturing jars. In this case 20 sealed jars are required.
2. Seal appropriate number of glass jars with aerial seal film and rubber band.
3. Autoclave the medium and the sealed glass jars. Name the jars as 14N-Light and 15N-Heavy accordingly.
4. Open the seal of the sterilized glass jars and pour the serialized medium into the glass jar until it reaches 1.5 cm above the bottom of the glass jar. This step MUST be undertaken in a laminar flow hood. Keep the seal film in the hood.
5. Dry the jars in the laminar flow hood for 2-3 hours.

5.4. Step C-E: Grow the sterilized seeds as two different populations.

1. Place the sterilized seeds in optimized density (4-16 seedlings per jar) gently on the agar surface using your own optimized technique. This step MUST be undertaken in a laminar flow hood.
2. Grow one plant population in 14N-Light PlantNitro® and the other plant population in 15N-Heavy PlantNitro® (isotope labeled) medium.
3. After planting, dry the jars in the laminar flow hood for 1 hour, then seal the jars with the autoclaved aerial seal film and rubber band, replace the rubber band if necessary.
4. Place the sealed jars in the growth chamber under optimized growth condition of your model plant.

5.5. Step F: Harvest the plant tissue

1. When the plants become mature, open the seal of the glass jars.
2. Pour approximately 50 ml of liquid nitrogen into the jar, wait for 5 seconds until the agar surface becomes frozen.
3. Harvest the tissue using a pre-cooled metal specular. Keep the tissue in a 50 ml conical centrifuge tube submerge in liquid nitrogen. Note: ALWAYS keep the tissue in liquid nitrogen during this step. The tissue obtained in this step can be stored for a few months at -80 ^{O}C.

5.6. Step G: Mixing of labeled and unlabeled samples

1. The tissue can be mixed in 1:1 mass ratio by weighing (Frozen using liquid nitrogen).
2. Or, the protein content can be mixed in 1:1 ratio after determined the protein concentration using protein assay methods such as DC assay.

5.7. Step H: Protein Extraction

Extract the protein from the tissue using NitroExtra™ protein extraction buffer (Cat. No. PEX-001-250ML) or other extraction buffers that can extract the protein and maintain the protein in fully denatured state. Refer to the manual of NitroExtra™ manual for details of the extraction process.

5.8. Step I: SDS gel separation

The protein has to be separated by SDS gel and digested by In-gel digestion using common SDS gel protocols available.

A general protocol for in-gel trypsin digestion is provided below. You may use any method of choice or a method recommended by your proteomics core facilities. For more information, refer to published reference sources (Coligan et al., 1998; Helmann et al., 1995).

Note: The digestion protocol given below is generally used for protein identification. If larger sequence coverage is desired, reduction and alkylation of peptides is recommended (Shevchenko et al., 1996). To avoid keratin contamination, always use clean lab-wares and wear gloves. Perform the in-gel digestion in a laminar flow hood.

1. Rinse the stained gel in water for 10 minutes to remove any particulate materials.
2. Excise the desired gel band from the stained gel. Mince the excised gel piece into smaller pieces (1 mm x 1 mm). Transfer the gel pieces to a clean microcentrifuge tube.
3. Add 500 µl 50% acetonitrile/25 mM ammonium bicarbonate (pH 8.0). Incubate at room temperature for 15-30 minutes for destaining the gel pieces. Vortex for 10 seconds and briefly centrifuge at maximum speed for 10 seconds. Discard the supernatant carefully without removing gel pieces.
4. Repeat Step 3 until the gel pieces are sufficiently destained.
5. Add 200 µl 100% acetonitrile to dehydrate the gel pieces.
6. Incubate for 5–10 minutes at room temperature. Vortex for 10 seconds and briefly centrifuge at maximum speed for 10 seconds. Discard the supernatant carefully without removing any gel pieces.
7. Dry the gel pieces in a centrifugal vacuum concentrator.
8. Add enough cold trypsin solution (10 ng/µl in 25 mM ammonium bicarbonate, pH 8.0) to cover the gel pieces.
9. Incubate on ice for at least 1 hour to allow the trypsin solution to penetrate the gel pieces. The cold temperature helps to prevent autolysis of the trypsin.
10. Incubate overnight at 37°C.
11. Add 50 µl 5% formic acid (FA), and incubate for 30 minutes at room temperature.
12. Vortex for 30 seconds, centrifuge at 14,000 x g for 1 minute, and collect the supernatant.
13. Add 50 µl 5% FA, 50% acetonitrile, and incubate for 30 minutes at room temperature.
14. Vortex for 30 seconds, centrifuge at 14,000 x g for1minute, and collect the supernatant, pooling it with the supernatant from Step 12.

15. Concentrate the supernatant using a centrifugal vacuum concentrator to ~5 µl. Do not allow the samples to dry out.
16. Submit your tryptic peptides to the proteomics core facility for analysis.

5.9. Step J: Mass Spectrometric Analysis

The tryptic peptides can be analyzed using the following MS analysis methods.

Important: When identifying and quantitating proteins using metabolic labeling technique, it is important to perform MS analysis using tandem MS instruments that are capable of performing MS/MS analysis.

For samples of low complexity, use MS fingerprinting techniques to identify and quantitate proteins.

For samples of moderate complexity, use MALDI-TOF MS analysis. We routinely use 4700 Proteomics Analyzer (MALDI-TOF/TOFTM instrument) from Applied Biosystems. Other instruments such as Bruker Reflex III (Bruker Daltonics) or Voyager-DETM STR MALDI TOF Workstation (Applied Biosystems) are also suitable.

For complex samples, use on-line or off-line LC-MS/MS or two- dimensional LC-MS/MS. You may use Micromass Q-Tof PremierTM Mass Spectrometer (Waters) or QSTARPulsar quadrupole TOF tandem MS (Applied Biosystems) equipped with a nanoelectrospray ion source or off- line separations followed by MALDI-TOF/TOF analysis.

For LC-MS analysis, the following gradients are recommended. If you optimize the LC-MS analysis with specific gradients that are suitable for your analysis, use the optimized gradients for your analysis. For samples with less complexity, use a gradient of 5-45% (v/v) acetonitrile in 0.1% formic acid (or TFA) over 45 minutes and then use a gradient 45-95% acetonitrile in 0.1% formic acid (or TFA) over 5 minutes. Note: Use 0.1% formic acid solution on ESI based instruments and 0.1% TFA solution on off-line LC-MS/MS analysis using MALDI-TOF/TOF.

For a complex sample, use a gradient of 5-45% (v/v) acetonitrile in 0.1% formic acid (or TFA) over 90 minutes or up to 120 minutes, and then use a gradient of 45-95% acetonitrile in 0.1% formic acid (or TFA) over 30 minutes or up to 60 minutes.

5.10. Step K: Protein identification with Mascot software suite

The data file obtained from MS/MS experiment is often multi-gigabyte in size. However, only a subset of the data, namely MS2 and their precursor MS1 spectra, are useful for peptide sequence identification. Instead of requiring the search engine to go through the entire raw file, it is always a good idea to convert the raw file into a search engine compatible format. If Mascot is used, for example, freeware like MSConvert can convert raw data file into Mascot Generic Format (MGF) which is hundreds of kilobytes in size and contains all the necessary information for peptide identification (Figure 1). MSConvert is bundled with ProteoWizard. To obtain MSConvert, visit the download page[1]of ProteoWizard and install the distribution package with vender reader support (Figure 2). If your computer is not up to date, you may be required to install NetFramework 4.0 when trying to install ProteoWizard (Figure 3). Download NetFramework 4.0 from Microsoft or follow the link here[2].

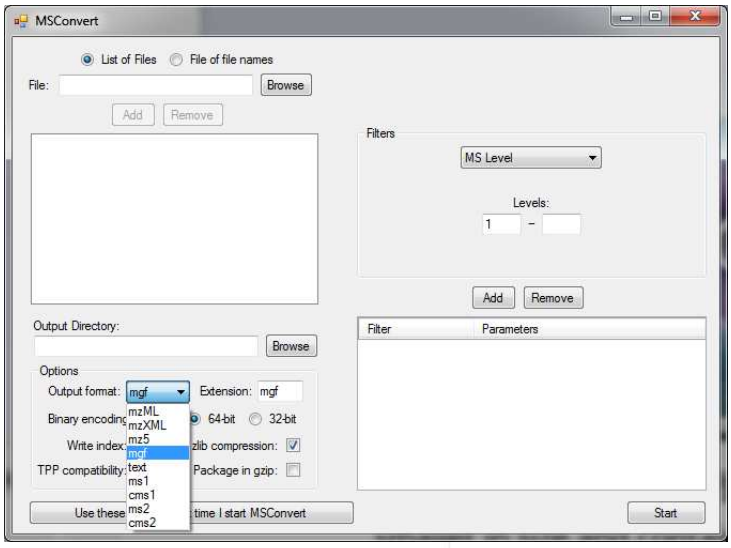


Figure 1 MSConvert is a freeware that convert raw data file generated by different brand of mass spectrometers into peptide search engine compatible format like MGF. This software also useful to produce mzML/mzXML universal MS data storage format. You can also extract a section of spectra from the raw data file by adding "Subset" filter.

[1] http://proteowizard.sourceforge.net/downloads.shtml
[2] http://www.microsoft.com/en-us/download/details.aspx?id=17718

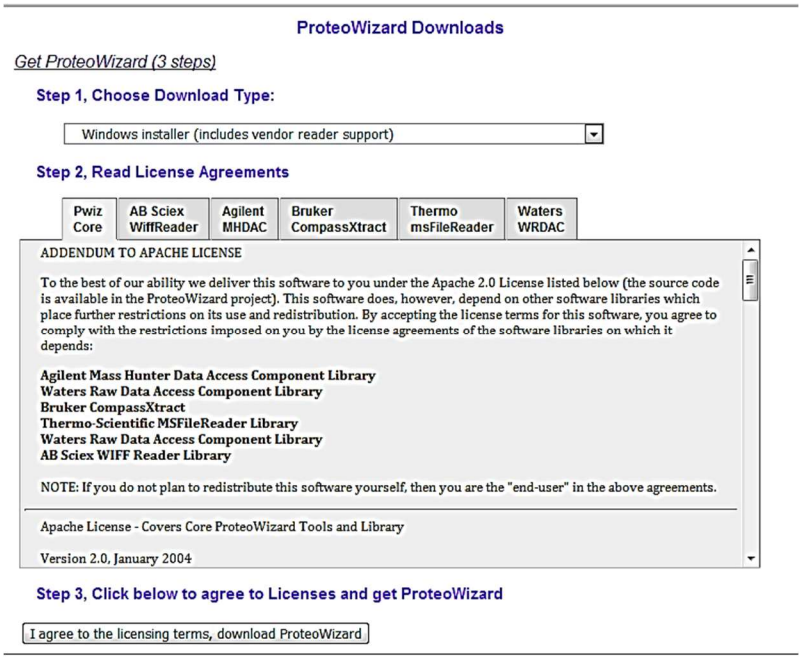

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Figure 2: Download MSConvert through ProteoWizard. Select the package contain vender reader support.

Figure 3: ProteoWizard requires NetFramework 4.0 installed in computer.

The converted MGF can be submitted into offline/online Mascot server for searching. Online Mascot search has limitation on the number of spectra that can be submitted at one time. When performing a database search, make sure correct parameters, related to your protein samples and mass spectrometer, are given. Example settings are shown in Figure 4.

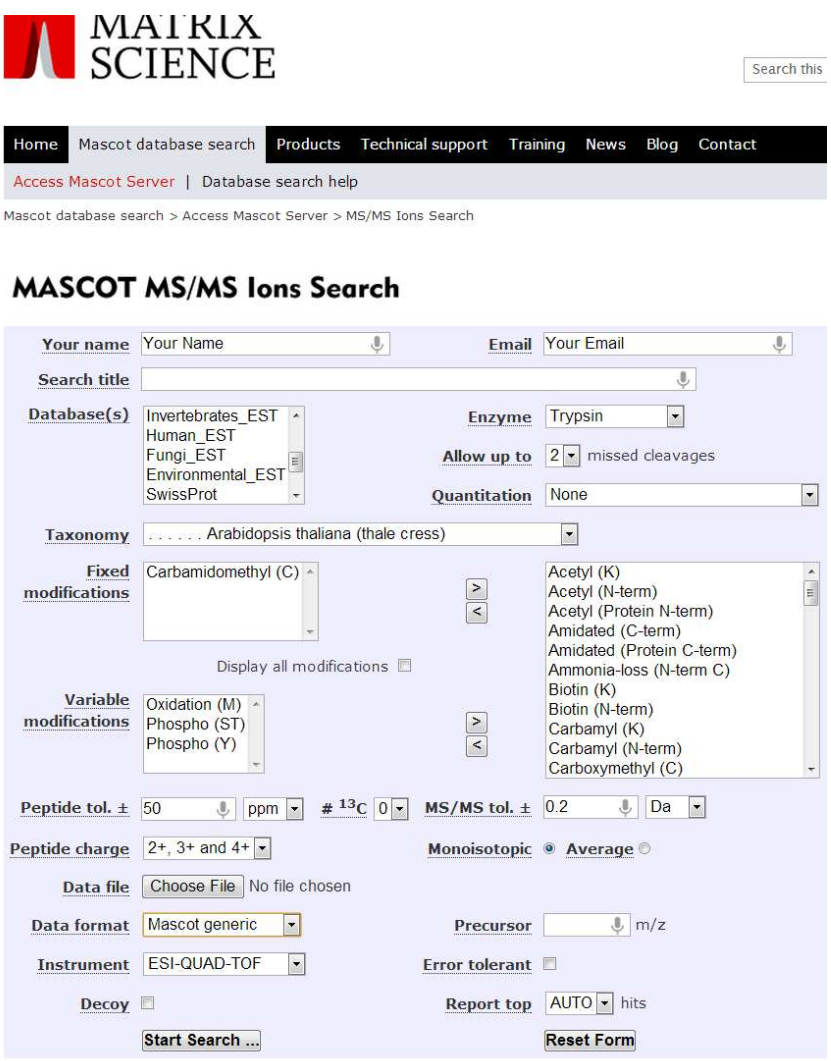

Figure 4: Example Mascot MS/MS Ions Search setting. For details of individual parameter please click their respective labels.

Typical online Mascot search takes about 1-2 minutes depending on the speed of your computers. Once the search completed, export the search result in DAT file format which will be used for subsequent peptide level quantification.

5.11. Step L: Protein quantitation with NitroQuant™ software

NitroQuant™ is software designed to calculate the ratio of unlabeled to labeled peptide level by taking heavy nitrogen incorporation rate into account (Equation 1). Currently, this software only supports windows platform and required computers to have the most updated JAVA installed. Administrative right is required to install the software.

$$\text{Ratio} = \frac{(14\text{N} - \text{Peptide})\ \text{Integrated Intensity Level}}{(15\text{N} - \text{Peptide})\ \text{Integrated Intensity Level}}$$

Equation 1 Relative peptide level calculation

To quantify peptide level, the program needs to read the raw MS data file from your hard disk. However, you must authorize the program to do so by choosing "Yes" in the authorization dialogue. The program may take longer time to start in the first time to setup proper environment for quantification. Once loading is completed, you can input your in-house incorporation rate in the parameter panel and then click "New Project". A new window will show up (Figure 5). Browse to the location of your raw data file/directory and also the Mascot search result file which is assumed to be stored in DAT format. The raw MS data file and Mascot search result file must store on the same folder and both of them must have the same file name, e.g. yourfilename.raw and yourfilename.dat.

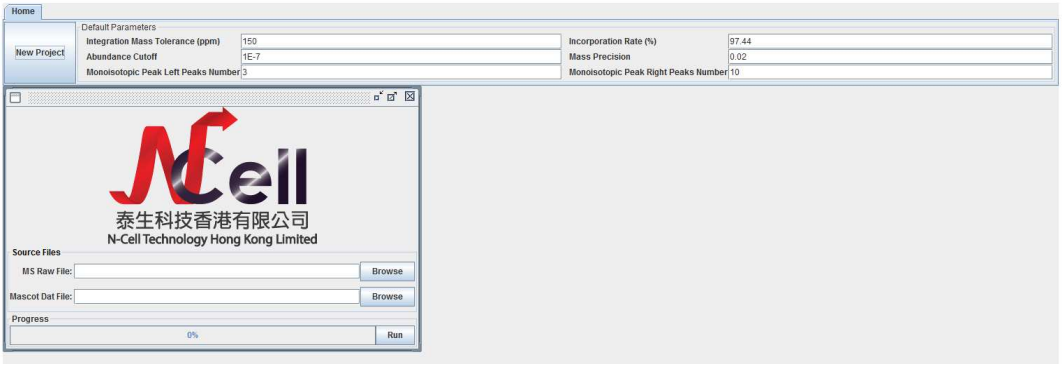

Figure 5: To start a quantification, browse the raw data file (.mzML/.mzXML) and Mascot search result file (.dat).

The program begins by converting the raw MS data and Mascot search result file into universal data formats which is mzXML and pepXML respectively. The time required for the conversion process depends on the original file size. The larger the raw file is, the longer the time it requires. The converted files will feed into the XPRESS algorithm of trans-proteomic pipeline to identify elution peaks. After knowing the range of each elution peak, incorporation rate will then be used to quantify relative peptide levels by N-Cell Tech custom analysis algorithm which takes the entire measured isotopic envelope into account.

The result of quantification is displayed in a table format (Figure 6). Each row represents a peptide and contains a ratio calculated using Equation 1. This ratio can be adjusted by changing the value of start/end scan number, charge, light and heavy M/Z (Figure 9, Figure 10, Figure 11, Figure 12 and Figure 13). Quantification result can be plotted in three ways, namely Intensity vs. M/Z (Figure 7), Intensity vs. Scan Number (Figure 8) and 3D plot. All the quantification results and data used for plotting can be

exported in excel format (Figure 14, Figure 15 and Figure 16). Each column can be hide or shown by toggling the checkbox in the top panel (Figure 17).

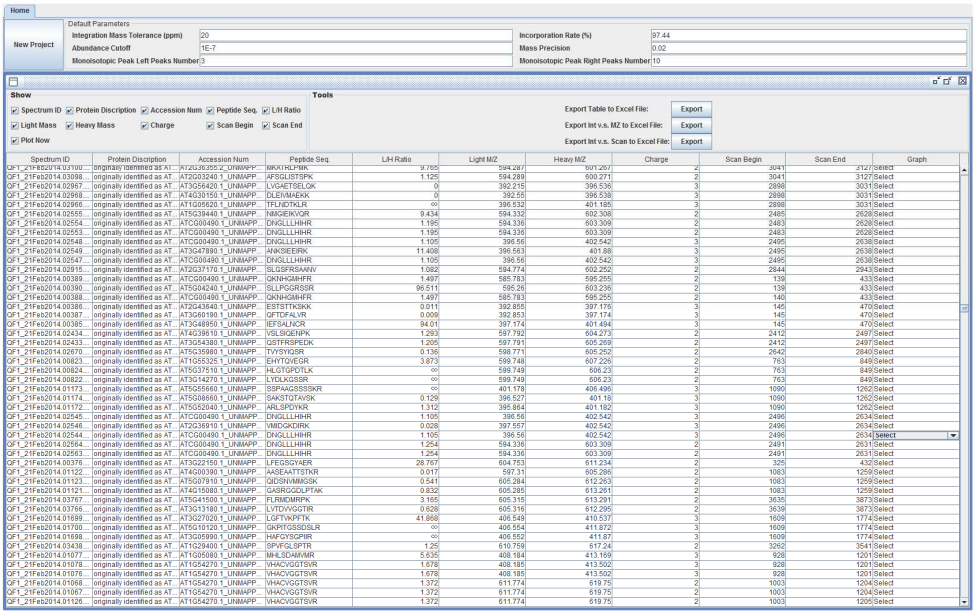

Figure 6: The result of 15N quantification is shown in table format.

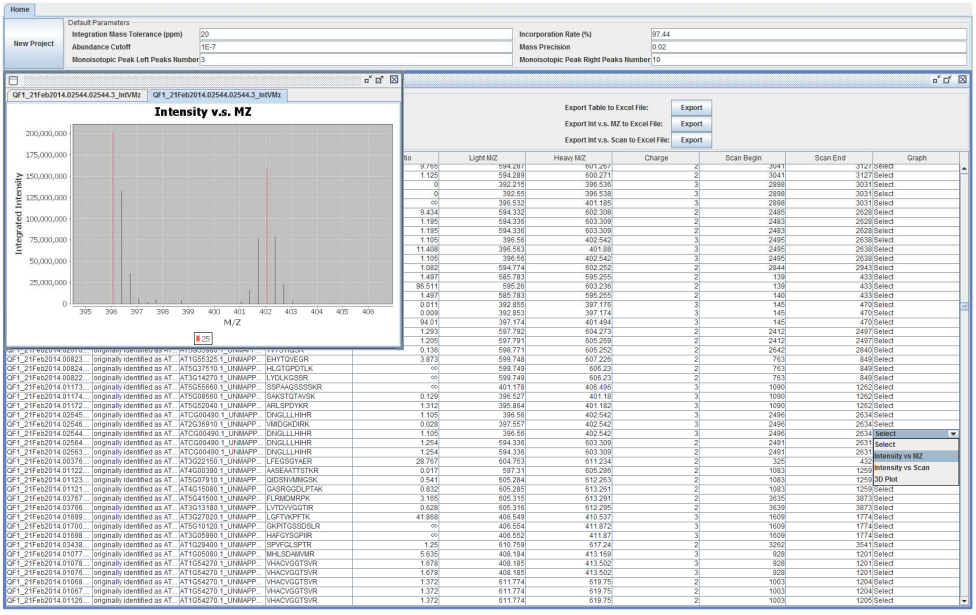

Figure 7: Intensity vs. M/Z plot. The intensity is an integrated value over the elution profile of the peptide.

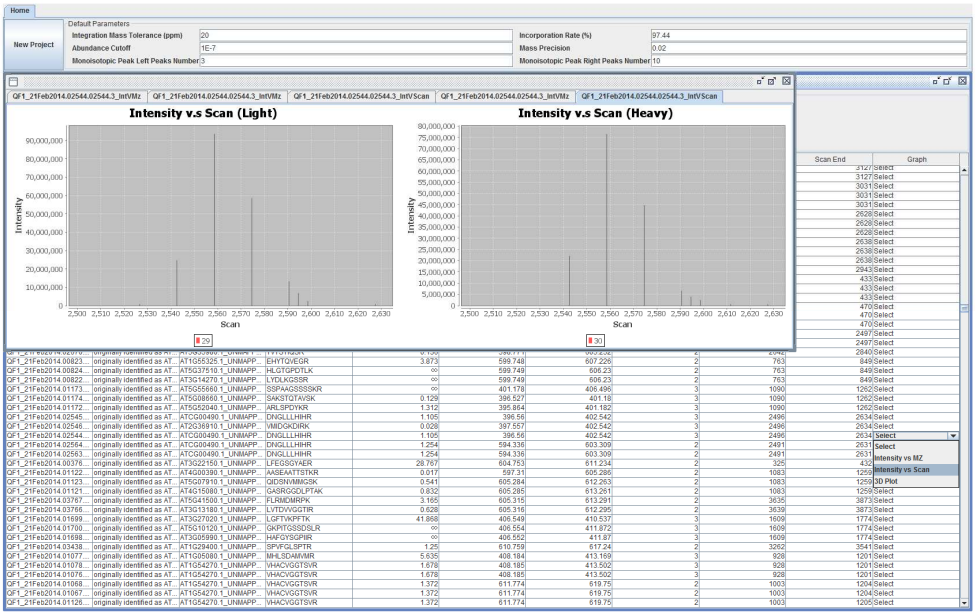

Figure 8: Intensity vs. Scan Plot.

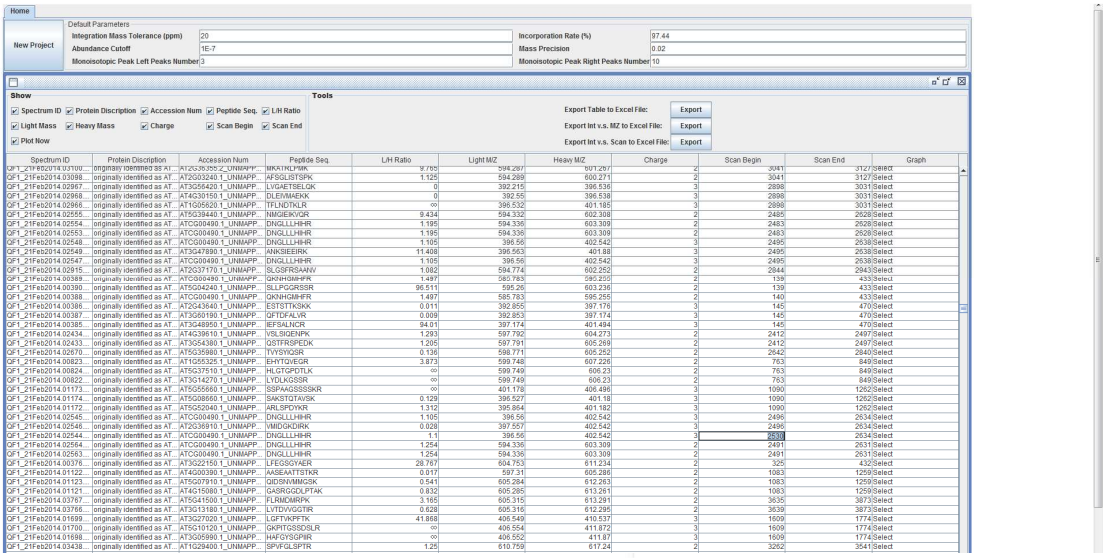

Figure 9: The ratio can be adjusted by changing the value of start/end scan number, charge, light and heavy M/Z.

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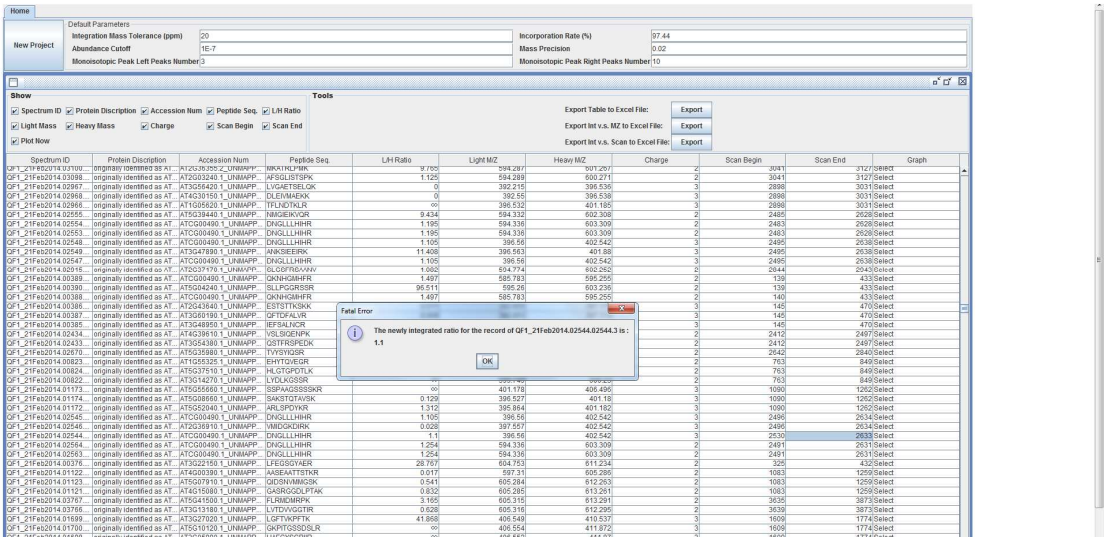

Figure 10: A dialogue showing the recalculated ratio after parameters change.

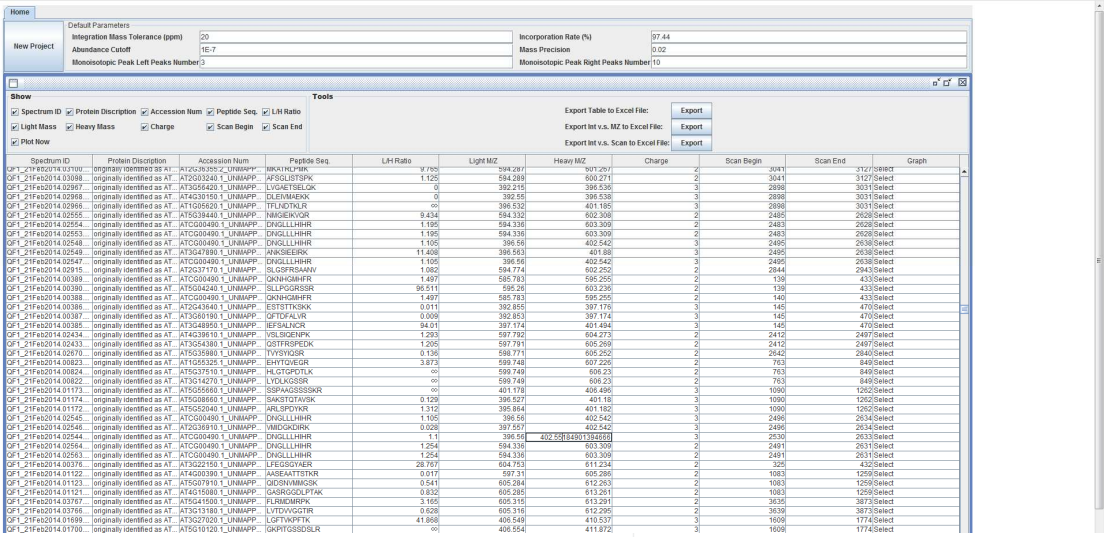

Figure 11: Light or heavy M/Z value can be changed.

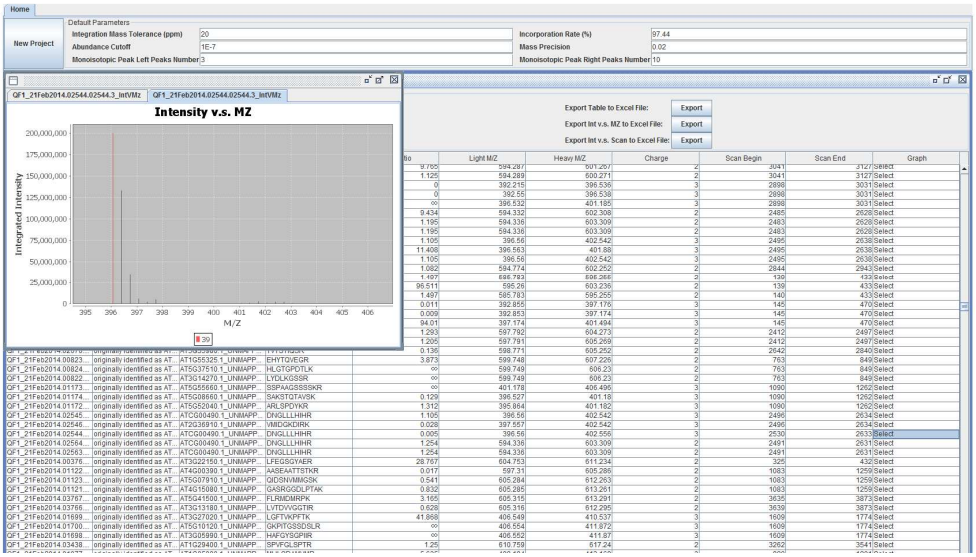

Figure 12: Graph will be updated automatically after parameter change.

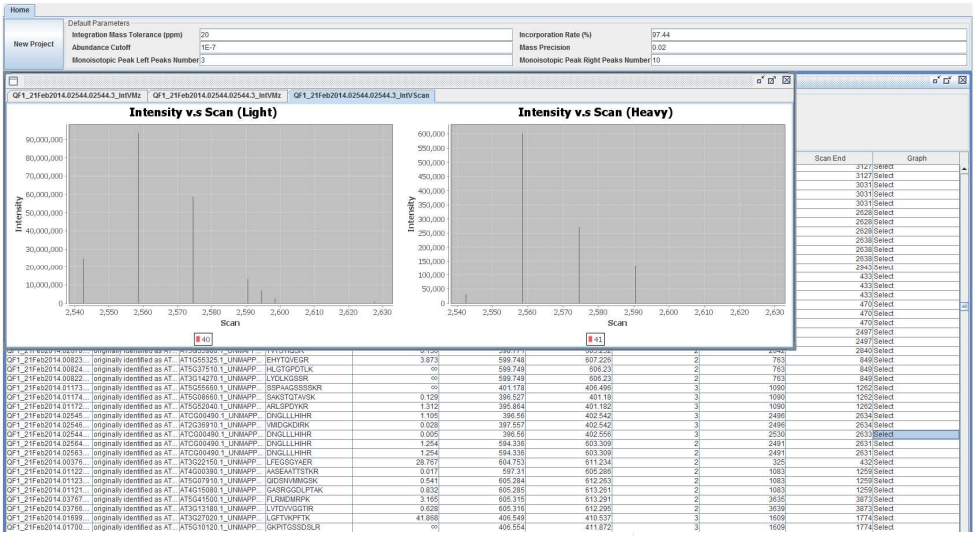

Figure 13: Graph will be updated automatically after parameter change.

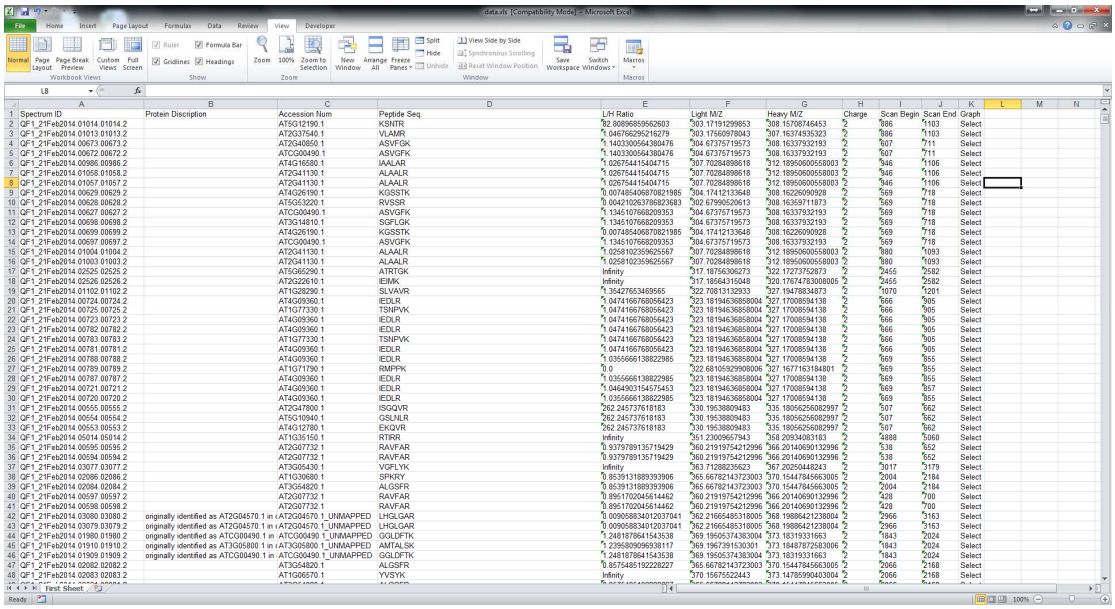

Figure 14: The data in the table can be exported.

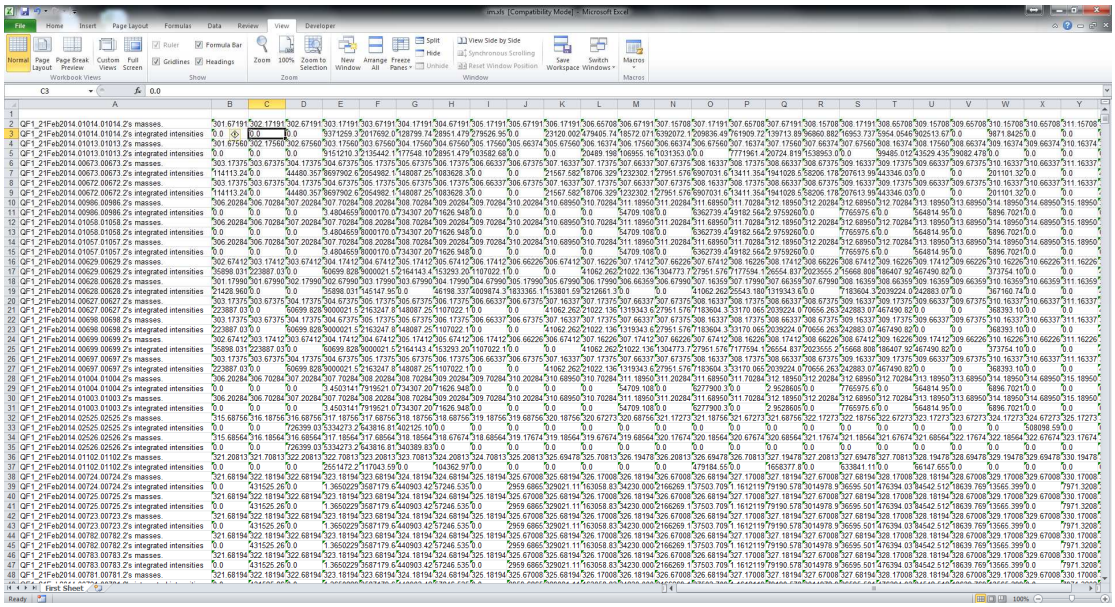

Figure 15: Data used for Intensity vs. M/Z plot can be exported.

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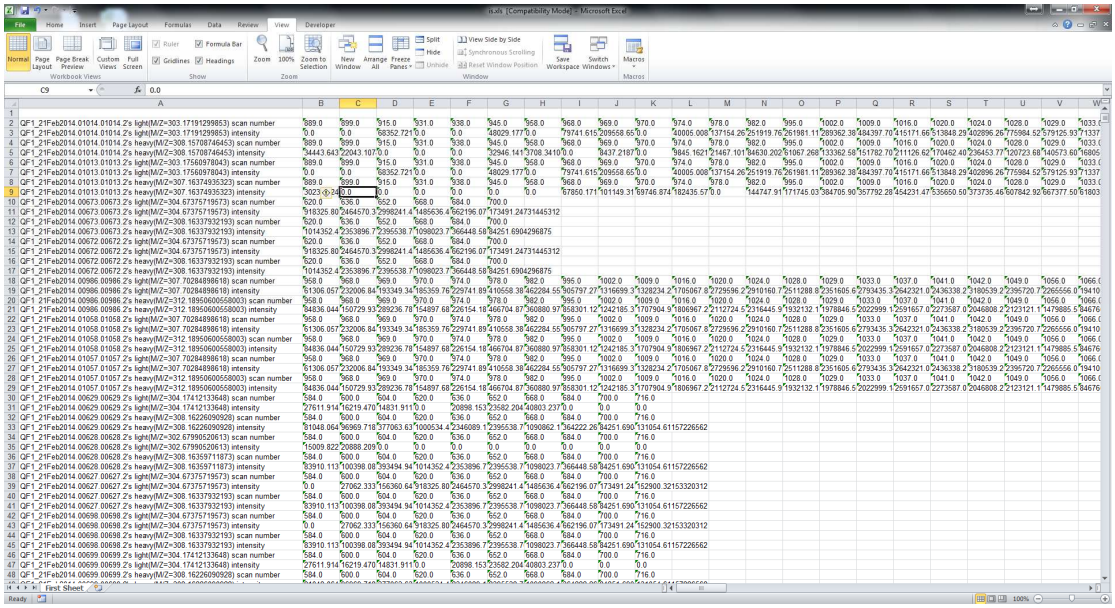

Figure 16: Data used for intensity vs. Scan Number plot can be exported.

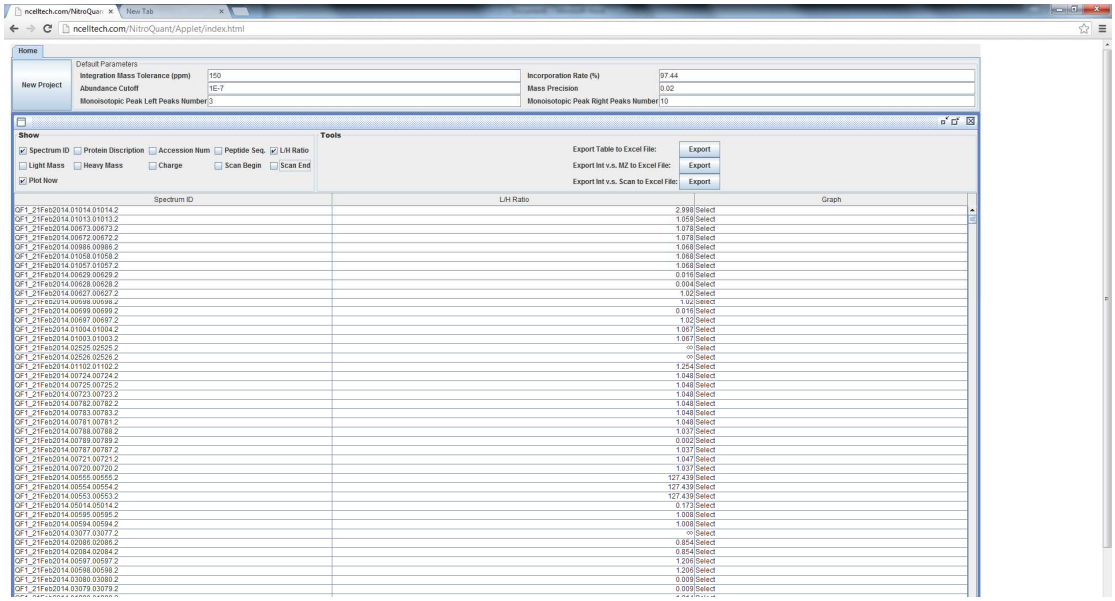

Figure 17: Column(s) can be shown or hide by toggling corresponding checkbox.

Should any technical support (including data analysis) is needed, please feel free to email us at support@ncelltech.com or call (852) 34603118.

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3. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) Mass Spectrometric Sequencing of Proteins from Silver-stained Polyacrylamide Gels. Anal. Chem 68, 850-858